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Abstract: Detection and protection of apoptosis, autophagy and neurovascular unit (NVU) are essentially important in understanding and treatment for ischemic stroke patients. In this study, we have conducted an in vivo optical imaging for detecting apoptosis and activation of matrix metalloproteinases (MMPs), then evaluated the profective effect of 2 package types of free radical scavenger edaravone (A and B) on apoptosis, autophagy and NVU in mice after transient middle cerebral artery occlusion (tMCAO). As compared to vehicle treatment, edaravone A and B showed a significant improvement of clinical scores and infarct size at 48 h after 90 min of tMCAO with great reductions of in vivo fluorescent signal for MMPs and early apoptotic annexin V activations. Ex vivo imaging of MMPSense 680 or annexin V-Cy5.5 showed a fluorescent signal, while which was remarkable different between vehicle and edaravone groups, and colocalized with antibody for MMP-9 or annexin V. Edaravone A and B ameliorated the apoptotic neuronal cell death in immunohistochemistry, and activations of MMP-9 and aquaporin 4 with reducing autophagic activations of microtubule-associated protein 1 light chain 3 (LC3) in Western blot. In this study, edaravone in both packages showed a similar strong neuroprotection after cerebral ischemia, which was confirmed with in vivo and ex vivo optical imagings for MMPs and annexin V as well as reducing cerebral infarct, inhibiting apoptotic/autophagic mechanisms, and protecting a part of neurovascular unit.

Dear Dr. Frank R. Sharp, and Irwin B. Levitan editor-in-chief,

I am writing this letter for submitting our revised version of a paper entitled “In Vivo Optical Imaging for Evaluating the Efficacy of Edaravone after Transient Cerebral Ischemia in Mice” for Research Report section of Brain Research. All of the content of this manuscript has been read and checked by all authors. All authors will take full responsibility for the data, the analyses and interpretation, and the conduct of the research. This manuscript has not been previously published, and it is not under simultaneous consideration by another journal. In addition, this manuscript has not been written by anyone else not named on the author list.

This is a report on *in vivo* and *ex vivo* optical imagings for evaluating the effect of edaravone after cerebral ischemia. We believe our manuscript expands that edaravone in both packages showed a similar strong neuroprotection after cerebral ischemia, which was confirmed with in vivo and ex vivo optical imagings for MMPs and annexin V as well as reducing cerebral infarct, inhibiting apoptotic/autophagic mechanisms, and protecting a part of neurovascular unit. We would be grateful if the manuscript could be reviewed and considered for a possible publication in ***brain Reseach*** as Regular Manuscript.

Thank you very much for your time and consideration.

Sincerely yours,

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***Research Highlights**

Reseach Highlights

- Efficacy of edaravone after transient cerebral ischemia can evaluate by in vivo optical imaging.
- Edaravone in both packages inhibited apoptosis.
- Edaravone in both packages inhibited autophagy.
- Edaravone in both packages protected a part of neurovascular unit.

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In Vivo Optical Imaging for Evaluating the Efficacy of Edaravone after Transient Cerebral Ischemia in Mice

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Abbreviations:

Atg8, autophagy-related genes 8; AQ-4, aquaporin 4; CBF, cerebral blood flow; CCD, charge-coupled device; ECL, enhanced chemiluminescent; HE, hematoxylin and eosin; LC3, microtubule-associated protein 1 light chain 3; LEL, lycopersicon esculentum lectin; MMP, matrix metalloproteinases; MMP-9, matrix metallopeptidase 9; MCA, middle cerebral artery; NIRF, near-infrared fluorescence; NAGO, N-acetylglucosamine; NVU; neurovascular unit; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; ROI, regions of interest; PS, phosphatidylserine; TBRs, target-to-background ratios; SC, sham control; tMCAO, transient middle cerebral artery occlusion; TUNEL, terminal deoxynucleotidyl transferasemediated dUTP-biotin in situ nick end labeling; TFI, transillumination fluorescence imaging; Ve, vehicle.

Abstract

Detection and protection of apoptosis, autophagy and neurovascular unit (NVU) are essentially important in understanding and treatment for ischemic stroke patients. In this study, we have conducted an in vivo optical imaging for detecting apoptosis and activation of matrix metalloproteinases (MMPs), then evaluated the profective effect of 2 package types of free radical scavenger edaravone (A and B) on apoptosis, autophagy and NVU in mice after transient middle cerebral artery occlusion (tMCAO). As compared to vehicle treatment, edaravone A and B showed a significant improvement of clinical scores and infarct size at 48 h after 90 min of tMCAO with great reductions of in vivo fluorescent signal for MMPs and early apoptotic annexin V activations. Ex vivo imaging of MMPSense 680 or annexin V-Cy5.5 showed a fluorescent signal, while which was remarkable different between vehicle and edaravone groups, and colocalized with antibody for MMP-9 or annexin V. Edaravone A and B ameliorated the apoptotic neuronal cell death in immunohistochemistry, and activations of MMP-9 and aquaporin 4 with reducing autophagic activations of microtubule-associated protein 1 light chain 3 (LC3) in Western blot. In this study, edaravone in both packages showed a similar strong neuroprotection after cerebral ischemia, which was confirmed with in vivo and ex vivo optical imagings for MMPs and annexin V as well as reducing cerebral infarct, inhibiting apoptotic/autophagic mechanisms, and protecting a part of neurovascular unit.

Key words: apoptosis, autophagy, edaravone, cerebral ischemia, in vivo imaging.

1. Introduction

Stroke is the leading cause of death and adult disability in worldwide. Ischemic stroke is a disturbance of cerebral circulation which generates oxygen free radicals, eventually relating to neuronal cell death during cerebral infarction and even in reperfusion (Flamm et al., 1978). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, synonyms MCI-186) is a free radical scavenger which originally showed a strong reduction of ischemic brain edema (Abe et al., 1988), and showed a clinical effect in petients with acute cerebral ischemia (Edaravone Acute Infarction Study Group, 2003; Ohta et al., 2009). Thus edaravone was approved by the Japanese government in 2001 as the first neuroprotective drug for acute ischemic stroke patients.

Cerebral ischemia induces both apoptosis (Renolleau et al., 1998; Abe K, 2000) and autophagy (Adhami et al., 2006; Shang et al., 2010). Annexin V detects an early apoptotic stage when phosphatidylserine (PS) is exposed on the surface of cells (Chopp et al., 1996), while terminal deoxynucleotidyl transferasemediated dUTP-biotin in situ nick end labeling (TUNEL) detects a late stage of apoptosis when DNA is degrading after brain ischemia (Li et al., 1995). Microtubule-associated protein 1 light chain 3 (LC3), a mammalian homologue of autophagy-related genes 8 (Atg8), is an important marker and effector of autophagy (Kirisako et al., 1999; Tian et al., 2010).

Recent reports suggest that NVU including matrix metallopeptidase 9 (MMP-9) and aquaporin 4 (AQ-4) played an important role after cerebral ischemia. MMP-9 is 92 kDa type IV collagenase, which is one of the matrix metalloproteinases and highly be

activated in the central nervous system during the inflammatory response after cerebral ischemia (del Zoppo GJ, 2010; Yamashita et al., 2009). AQ-4 is one of 13 known water channel aquaporins in mammals, which is mainly located in the astrocyte foot process and playing a role in the post-ischemic brain edema after ischemia (Papadopoulos et al., 2002; Lo et al., 2005).

In previous reports, series of fluorescent probe were used for in vivo optical imaging after cerebral ischemia, such as CD40, annexin V, MMP and LC3 (Klohs et al., 2008 and 2009; Liu et al., 2010; Tian et al., 2010). The protective effect of edaravone has not been documented in details on this in vivo imaging relating with apoptosis, autophagy and NVU. Edaravone has 2 types of package made by glass or polyethylene, both of which have a respective advantage and disadvantage in clinical setting, but the effect of their different package has not been directly compared. In this study, therefore, we attempted to examine in vivo optical imaging for MMPs and annexin V in relation to apoptosis, autophagy and NVU, and evaluate the efficacy of either package of edaravone after transient cerebral ischemia in mice.

2. Results

2.1 Moto coordination

As compared to sham control (SC) animals, animals with vehicle (Ve) treatment showed great reductions of the Rota-rod treadmill scores at 0 h (13.22 ± 6.71, $^{**}p<0.01$

vs SC) and 48 h (20.01 ± 6.07, **p<0.01 vs SC) after tMCAO. Treatment of the ischemic animals with edaravone A or B improved the Rota-rod scores at 48 h after tMCAO (27.40 ± 3.49 and 27.41 ± 3.77, *p<0.05 vs SC, #p<0.05 vs Ve) (Fig.1).

2.2 In vivo and ex vivo imaging

Fluorescence imaging analyzer scarecely detected a fluorescent signal over the head with skin in any groups of living mice with MMPSense 680. With removal of head skin, a weak fluorescent signal was detected over the ischemic hemisphere in vivo in vehicle treated animals at 48 h after tMCAO (Fig. 2a). Removal of skull bone made such a fluorescent signal more evident especially over the ischemic hemisphere at 48 h in vehicle and edaravone (A and B) treated animals in vivo (Fig. 2b). Ex vivo fluorescent signals were more clearly detected after removing their brains (Fig. 2c). The regions of interest (ROI) were selected over the both hemispheres, and target-to-background ratios (TBRs) were calculated by specific value of the average intensity of the ROI of the right and left hemispheres. Fig. 2d showed that TBRs of MMPSense 680 with the skull bone was 1.01 ± 0.01 in SC group, 1.36 ± 0.01 in Ve group (*p<0.05 vs SC), 1.17 ± 0.01 in edaravone A group (*p<0.05 vs SC, #p<0.05 vs Ve), and 1.15 ± 0.01 in edaravone B group (*p<0.05 vs SC, #p<0.05 vs Ve). Fig. 2e showed that TBRs of MMPSense 680 after removal of skull bone was 1.01 ± 0.01 in SC group, 1.61 ± 0.01 in Ve group (*p<0.05 vs SC), 1.31 ± 0.03 in edaravone A group (*p<0.05 vs SC, #p<0.05 vs Ve), and 1.30 ± 0.02 in edaravone B group (*p<0.05 vs SC, #p<0.05 vs Ve).

In the mice with injection of annexin V-Cy5.5, a weak fluorescent signal was detected over the ischemic hemisphere in vivo in vehicle treated animals at 48 h after tMCAO (Fig. 2f). Removal of skull bone made such a fluorescent signal more evident especially over the ischemic hemisphere at 48 h in vehicle and edaravone (A and B) treated animals in vivo (Fig. 2g). Ex vivo fluorescent signals were more clearly detected after removing their brains (Fig. 2h). Fig. 2i showed that TBRs of annexin V-Cy5.5 with the skull bone was 1.00 ± 0.03 in SC group, 1.21 ± 0.03 in Ve group (*****p<0.05 vs SC), 1.14 ± 0.03 in edaravone A group (*****p<0.05 vs SC), and 1.12 ± 0.04 in edaravone B group (*****p<0.05 vs SC). Fig. 2j showed that TBRs of annexin V-Cy5.5 after removal of skull bone was 1.00 ± 0.04 in SC group, 1.53 ± 0.04 in Ve group (*****p<0.05 vs SC), 1.22 ± 0.04 in edaravone A group (*****p<0.05 vs SC, #p<0.05 vs Ve), and 1.22 ± 0.05 in edaravone B group (*****p<0.05 vs SC, #p<0.05 vs Ve).

2.3 Cerebral infarct volume

In contrast to no lesions in either hemisphere of SC group, the total infarct volume of Ve, edaravone A and B groups were 91.38 ± 4.31 mm^3, 67.68 ± 11.47 mm^3 (#p<0.05 vs Ve) and 66.08 ± 10.70 mm^3 (#p<0.05 vs Ve), respectively (Fig. 3). Subregional analysis of the cerebral infarct showed that infarcts in the cerebral cortex and caudate were 63.54 ± 5.18 and 27.84 ± 5.52 mm^3 in Ve group, and that the edaravone A and B groups showed a smaller infarct volume in the cerebral cortex (47.44 ± 11.70 mm^3 and 46.46 ± 6.87 mm^3, respectively, #p<0.05 vs Ve) and caudate (20.24 ± 2.44 mm^3 and

19.62 ± 5.84 mm^3, respectively, #p<0.05 vs Ve).

2.4 The effect of anti-apoptotic and anti-autophagic effects of edaravone A and B

Although TUNEL staining was negative in the SC brain sections (Fig. 4a), tMCAO produced a large number of TUNEL positive cells at 48 h after the reperfusion in Ve group (Fig. 4b). Treatment of ischemic mice with edaravone A or B greatly reduced the number of TUNEL positive cells (Fig. 4c and d). Quantitative measurement of such TUNEL positive cells were shown in Fig. 4f, where the number of TUNEL positive cells in Ve, edaravone A and B groups were 1007.84 ± 110.32 cells/mm^2, 550.95 ± 60.54 cells/mm^2 (#p<0.05 vs Ve) and 544.24 ± 80.63 cells/mm^2 (#p<0.05 vs Ve), respectively.

Western blot analysis showed that a band of LC3-I was clearly present but scarecely of LC3-II in SC brain (Fig. 4e, SC). After tMCAO, the expression of LC3 (both I and II) was strongly induced in Ve group, and these strong inductions were greatly reduced in the ischemic mice treated with edaravone A or B (Fig. 4e). The amounts of LC3-I and II (relative to β-tubulin) were shown in Fig. 4g: SC group (0.23 ± 0.05 and 0.09 ± 0.01), Ve group (0.31 ± 0.06, **p<0.05 vs SC, 0.16 ± 0.02, **p<0.05 vs SC), edaravone A group (0.20 ± 0.04, #p<0.05 vs Ve, 0.13 ± 0.01, #p<0.05 vs Ve), edaravone B group (0.22 ± 0.03, #p<0.05 vs Ve, 0.13 ± 0.01, #p<0.05 vs Ve), respectively.

2.5 Protective effect of edaravone on blood brain barrier

Antibody for MMP-9 showed an immnohistochemical background as red fluorescent signal in 20 μm sections (Fig. 5a), which became much stronger in the ischemic hemisphere of vehicle treated mice (Fig. 5b, arrowheads). Treatment of ischemic mice with edaravone A or B significantly reduced such a MMP-9 immnohistochemical fluorescent signal (Fig. 5c and d, respectively). Double immunofluorescent analysis showed that colocalization of such an activated MMP-9 with N-acetylglucosamine (NAGO) (Fig. 5f) with much reduction of such a colocalization by edaravone A (Fig. 5g) or B (Fig. 5h). Immunofluorescent study of the ex vivo brain sections (intravenously injected MMPSense 680 or annexin V-Cy5.5) with an antibody for MMP-9 or annexin V showed a colocalization (Fig. 5k or 5n) of MMPSense 680 (Fig. 5j) or annexin V-Cy5.5 (Fig. 5m) and exogenous antibody for MMP-9 (Fig. 5i) or annexin V (Fig. 5l). Western blot analysis showed a great expression of MMP-9 and AQ-4 in Ve group after tMCAO and their reductions in edaravone A and B groups (Fig. 5o and p). Quantitative analysis of the amount of MMP-9 (relative to β-tubulin) in Western blot was as follows (Fig. 5q): 0.11 ± 0.03 in SC group, 0.68 ± 0.05 (**p<0.01 vs SC) in Ve group, 0.43 ± 0.03 (*p<0.05 vs SC, #p<0.05 vs Ve) in edaravone A group, and 0.45 ± 0.04 (*p<0.05 vs SC, #p<0.05 vs Ve) in edaravone B group. Quantitative analysis of the amount of AQ-4 (relative to β-tubulin) in Western blot was as follows (Fig. 5r): 0.23 ± 0.02 in SC group, 0.63 ± 0.07 (*p<0.05 vs SC) in Ve group,

0.50 ± 0.04 (*****p<0.05 vs SC, #p<0.05 vs Ve) in edaravone A group, and 0.49 ± 0.05 (*****p<0.05 vs SC, #p<0.05 vs Ve) in edaravone B group.

3. Discussion

We have shown that edaravone, a free radical scavenger, protected the brain against oxidative stress after cerebral ischemia (Abe et al., 1988; Zhang et al., 2004; Yamashita et al., 2009). Under normal condition, the control and modulation of local cerebral blood flow depends on neurovascular coupling (Nedergaard et al., 2003). Under ischemic condition, some components of the NVU are damaged, which then worsen the original damage of the brain. Apoptosis is one major mechanism contributing neuronal degeneration after ischemic insult (Linnik et al., 1995; Abe K, 2000), which may lead a deferred damage after ischemic stroke (Zhang et al., 2010). Our recent findings suggest that autophagy is another major mechanism involving in ischemic brain damage (Shang et al., 2010; Tian et al., 2010).

In the present study, we first showed a reduction of in vivo optical signals by edaravone A and B relating to MMP and annexin V activations (Fig. 2). The signal of MMPSense was strongly detected at 48 h after tMCAO (Fig. 2a - e). The effect of edaravone in the present study on MMP-9 (Fig. 5) is compatible with our previous reports (Yamashita et al., 2009; Lukic-Panin et al., 2010). The in vivo imaging of annexin V-Cy5.5 showed a peak of fluorescent signal for apoptosis at 48 h in our recent

report (Liu et al., 2010). In the present study, edaravone A and B treatments showed a significant reduction of such an apoptotic in vivo fluorescent signal at 48 h after tMCAO (Fig. 2f - j). As shown in Fig. 1, 3 and 4, edaravone improved clinical scores, reduced infarct volume, and ameliorated both apoptosis and autophagy, suggesting that the clinical scores and infarct volume may be associated with post-ischemic edema (Abe et al., 1988), apoptosis and autophagy (Abe K, 2000; Tian et al., 2010). In a recent report, we described that annexin V and TUNEL are the markers for early or late stage of apoptotic cells, respectively (Liu et al., 2010). Combined with such a previous report, our present in vivo imaging (Fig. 2f - j) and our TUNEL (Fig. 4f) studies supported the evidence that edaravone A and B had a strong anti-apoptotic effect. Similar to our previous reports (Shang et al., 2010; Tian et al., 2010) and others (Adhami et al., 2006), autophagy was also involved in the post-ischemic neuronal damage, which was ameliorated by edaravone A and B (Fig. 4e, g).

NVU provides a framework for bidirectional communication between neurons and their supporting microvessels with astrocytes interface (del Zoppo GJ, 2010; Yamashita et al., 2009). MMP-9, which is also called gelatinase B, is highly activated in the central nervous system during the inflammatory response after cerebral ischemia (del Zoppo GJ, 2010). The present study suggests that edaravone A and B protected a part of NVU by ameliorating MMP-9 activation (Fig. 5g, h), similar to our previous report (Yamashita et al., 2009). AQ-4 is a water channel in the central nervous system, which is expressed primarily in astrocytes foot processes and takes a part in NVU (Nagelhus et al., 2004). Edaravone reduced the activation of AQ-4 in mice brains after tMCAO (Fig. 5r) similar

to a previous report (Kikuchi et al., 2009). The good colocalization of the signal of MMPSense 680 or Cy5.5 and exogenous MMP-9 or annexin V antibody proved that the fluorescent signal detected in vivo animals and ex vivo sections actually represent MMPs and annexin V.

Edaravone A and B are MCI-186 (Formula: $C_{10}H_{10}N_2O$) in different packages, hence both edaravone A and B showed the similar neuroprotective effect regardless of the package (Fig. 1 - 5). Edaravone A is a solution dissolved in saline with L-cystein as a stabilizer in glass ampule, while edaravone B is a solution dissolved in saline with citrate as a stabilizer and sticky-loss preventor in polyethylene tube. The present study showed that edaravone A and B showed a similar effect as the same main ingredient regardless of package and stabilizer additives.

In summary, a free radical scavenger edaravone in both packages showed a similar strong neuroprotection of the brain after cerebral ischemia, which was confirmed with in vivo and ex vivo optical imagings for MMPs and annexin V as well as reducing cerebral infarct, inhibiting apoptotic/autophagic mechanisms, and protecting a part of NVU. Thus either package of edaravone could be useful for choosing the treatment in stroke clinics.

4. Materials and Methods

4.1 Surgical preparation

Adult 8-week-old mice (male, 35-37g) were used in this study. Wild-type ICR mice were purchased from SLC (Shizuoka, Japan). During surgery, the mice were anesthetized with a nitrous oxide/oxygen/isoflurane mixture (69/30/1%) administered through an inhalation mask. Rectal temperature was maintained at 37.0°C by placing the animals on a heating pad (model BMT-100; Bio Research Center, Nagoya, Japan). A laser Doppler flowmeter probe (model ALF21; Advance, Tokyo, Japan) was attached to the surface of the ipsilateral cortex to monitor regional cerebral blood flow (CBF). Middle cerebral artery occlusion (MCAO) was induced by the intraluminal filament technique reported previously (Abe et al., 1992; Yamashita et al., 2006; Lukic-Panin et al., 2007). In brief, the right carotid bifurcation was exposed, and a silicone-coated 8-0 filament was then inserted through the common carotid artery and gently advanced (9.0-10.0 mm) to occlude the middle cerebral artery (MCA). After 90 min of transient occlusion, CBF was restored by removed of the nylon thread. The SC group underwent the exposure of common carotid artery without subsequent MCAO (n=5). The experimental protocol and procedures were approved by the Animal Committee of the Okayama University Medical School of Medicine.

Edaravone is basically an acidic powder (pH 3.0-4.5) with an osmotic ratio of about 1 to physiological saline (0.9% NaCl) and is usually hard to be dissolved in H_2O or diethyl ether. Edaravone package A (edaravone A) is a solution in 20 ml of glass ampule, including 30mg of edaravone and a small amount of additives such as NaCl (135 mg), L-cystein (10 mg), and $NaHSO_2$ (20 mg) as a stabilizer, and a very small amount of NaOH as pH neutralizer. On the other hand, edaravone package B (edaravone

B) is a solution in 20 ml of polyethylene tube, including 30 mg of edaravone and a small amount of additives such as NaCl (150 mg), citrate (50 mg), $NaHSO_2$ (20 mg), and NaOH (7.3 mg) as a stabilizer, and a very small amount of NaOH as pH neutralizer. Polyethylene tube is safer than glass ampule for medical staff in emergent stroke clinic, but edaravone sticks the inside wall of polyethylene tube if the stabilizer for edaravone A was used, resulting in 50% loss of edaravone. Thus the stabilizer for edaravone B improves such a sticky loss to less than 5% with changing L-cystein to citrate. With these 2 types of package (1.5 mg/ml), edaravone was diluted into a concentration of 0.3 mg/ml with physiological saline, and injected as shown below.

After tMCAO, the mice were randomized into 3 groups and were subjected to injection with vehicle (n=5), edaravone A (n=5) or edaravone B (n=5), respectively. The intravenous injections were performed 3 times at immediately, 8 h and 24 h after tMCAO with 0.1 ml/10 g mice bodyweight, and the animals were sacrificed at 48 h just after taking data of in vivo image. The total dose of edaravone A and B was 9 mg/kg mice during 48 h of the present experiment, which was 3.75 times higher amount than clinical dose for stroke patients during initial 48 h (2.4 mg/kg patients).

All the animals were subjected to Rota-rod treadmill test (MK-610A, MUROMACHI KIKAI CO., LTD) just and 48 h after tMCAO. On the test, the animals were placed on the rotating rod (10 rpm) and timed when they fall off the shaft according to our previous report (Abe et al., 1997; Ohta et al., 2008).

4.2 In vivo imaging system and in vivo imaging

For in vivo imaging, we used the macro fluorescence imaging system (MVX 10 Macro View, Olympus, Japan). For excitation of fluorescent compounds, an intensity-controlled laser diode emitting at 682 nm was used. The fluorescence emission at 721 nm were collected by a charge-coupled device (CCD) camera (Digital camera C10600 ORCA^{R}-R^{2}, Hamamatsu, Japan) with the acquisition times 800 ms. The transillumination fluorescence imaging (TFI) were analyzed by MetaMorph Version 7.5 image analysis software.

Mice were subjected to in vivo imaging at 48 h after the tMCAO. The fluorescent compounds, MMPSense 680 (VisEn Medical Inc., USA) or annexin V-Cy5.5 (Catalog Number 559935, Becton, Dickinson and Company, Japan) were used in this experiment for testing matrix metalloproteinases (MMPs including MMP-2, -3, the key -9 and -13) and early apoptosis, respectively. MMPSense 680 (300 μl) or annexin V-Cy5.5 (200 μl) were intravenously injected at 12 h before (i.e., 36 h after tMCAO) the in vivo imaging. For in vivo imaging, the mice were anesthetized with a nitrous oxide/oxygen/isoflurane mixture (69/30/1%) administered through an inhalation mask, and the near-infrared fluorescence (NIRF) images were observed by a macro fluorescence imaging system described above with or without the head skin or skull bone. The SC group underwent the same performance only.

After taking in vivo image, the mice brains were removed under deep anesthesia with pentobarbital (40 mg/kg, i.p.), and ex vivo imaging for MMP was quickly

performed in the similar way as above. The brains were then immediately frozen in powdered dry ice. The sections of 20 μm thickness were respectively cut on a cryostat at -18°C, and collected on glass slides for histological analysis.

4.3 Infarct volume measurement

For quantitative analysis of infarct volume, the fresh-frozen 20 μm of mice brain sections were stained with hematoxylin and eosin (HE) and observed with a light microscope (Olympus BX-51; Olympus Optical). The area of the infarct was measured in 5 sections by pixel counting using a computer program of Photoshop 7.0, and the volume was calculated.

4.4 Single immunofluorescence analysis

The fresh-frozen 20 μm sections of the mice brain with MMPSense or annexin V-Cy5.5 injection were used for single immunofluorescent analysis. The sections were fixed with formaldehyde for 30 min, and then rinsed 3 times in phosphate-buffered saline (PBS; pH 7.4). After blocking with 5% bovine serum albumin for 1 h, the slides were incubated for 16 h at 4°C with the first antibody: goat polyclonal to MMP-9 or rabbit polyclonal to annexin V (1:100, Abcam) in PBS containing 5% bovine serum albumin and 0.3% Triton X-100. To confirm the specificity of the primary antibody, a

set of sections was stained in a similar way without primary antibodies. The sections were then washed and incubated for 2 h with the second antibody: Alexa Fluor 488 donkey anti-goat IgG or Alexa Fluor 488 donkey anti-rabbit IgG (A21206, Invitrogen) at 1:500. The slides were then covered with VECTASHIELD mounting medium with 4', 6'-diamidino-2-phenylindole (Vector Laboratories). The treated sections were scanned with Olympus BX51 (Japan). The CCD camera was Digital Camera C10600 (ORCA^{R} -R^{2}, Hamamatsu, Japan). The filter U-MNIBA2 and U-DM-CY5.5 (Olympus, Japan) were used. Acquisition times for the detection of Cy5.5 were 800 ms, and acquisition times for the detection of the alexa 488 were 1 s.

4.5 TUNEL staining

In accordance with our previous reports (Abe et al., 1997; Liu et al., 2010), TUNEL study was performed with the mice brain sections (20 μm) using a kit (Roche, Nonnenwald, Germany), which detects double-strand breaks in genomic DNA with diaminobenzidine.

4.6 Double immunofluorescence analysis

Double immunofluorescence studies were performed for N-acetylglucosamine (NAGO) plus MMP-9. The staining steps were the same as the above staining,

Lycopersicon esculentum lectin (LEL) is a glycoprotein with specific affinity for NAGO, which is expressed in mature vascular endothelial cells. The staining steps were the same as our previous report (Deguchi et al., 2006; Liu et al., 2010). Each dilution of the first antibody was as follows: biotinylated LEL (Vector laboratories) at 1:200, rabbit polyclonal antibody to MMP-9 (Abcam) at 1:100. The second antibodies were Alexa Fluor 588 donkey anti-goat IgG at 1:500 (Invitrogen) plus FITC avidin D (Vector laboratories). The slides were covered with VECTASHIELD Mounting Medium with 4', 6'-diamidino-2-phenylindole (Vector Laboratories). The treated sections were scanned with a confocal microscope equipped with an argon and HeNe1 laser (LSM-510; Zeiss, Jena, Germany). Sets of fluorescent images were acquired sequentially for the red and green channels to prevent crossover of signals from green to red or from red to green channels.

4.7 Western blot analysis

Tissue samples from the infarct cerebral hemisphere were homogenized with a homogenizer in lysis buffer (50 mM Tris-HCl, pH 7.2, 10% glycerol, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, and protease inhibitors). The homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and the total protein content determined using the Lowry assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (30 μg) were electrophoresed on a 12% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The

membranes were incubated in 5% skimmed milk in tris-buffered saline with 0.05% Tween 20 (TBS buffer) at room temperature for 2 h to block nonspecific binding. Then the membranes were probed with primary antibodies overnight at 4 °C, respectively. The primary antibodies were as follows: goat anti-mouse MMP-9 antibody (af909, Research & Diagnostics Systems, Inc.) diluted at 1:1000, rabbit polyclonal anti-aquaporin 4 antibody (ab46182, Abcam, Cambridge, UK) diluted at 1:1000 and rabbit polyclonal to LC3 (ab48394, Abcam, Cambridge, UK) diluted at 1:1000. The membranes were washed three times for 10 min each with TBS. Immunoblots was then incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h at ambient temperature. Immunodetection was performed with an enhanced chemiluminescent (ECL) substrate (Pierce, Rockford, IL, USA). After ECL detection, the membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.7; 2% SDS; 0.7% β-mercaptoethanol) at 60 °C for 30 min, and then reprobed with a monoclonal anti-β-tubulin antibody (1:5000; Sigma) as a loading control for protein quantification. The signals were quantified with a lumino-image analyzer (LAS 1000-Minutesi; Fuji Film, Tokyo, Japan), and we carried out densitometry analysis using Scion Image Beta 4.02 software. Quantitative results were obtained by measuring the optical density of each band and were expressed as the ratio of each targeted protein to β-tubulin expression.

4.8 Quantitative analysis

To evaluate the results of TUNEL staining, single immunohistochemical analysis and double immunofluorescent analysis quantitatively, the positively stained cells were counted in the cerebral cortex at the boundary zone in 5 coronal sections per brain. All data of staining, immunohistochemical and Western blot are expressed as means ± SD. The t-test with Post-hoc test was used for each evaluation (Deguchi et al., 2006).

Acknowlegment

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Figure legends

Figure 1. Rota-rod scores at 0 h and 48 h after tMCAO. ****p*<0.01 vs SC, **p*<0.05 vs SC, #*p*<0.05 vs Ve.

Figure 2. In vivo imaging of MMPSense 680 signal (a) with removal of head skin or (b) removal of skull bone, and (c) ex vivo imaging of the brain. (d, e) TBRs of MMPSense 680 showed that Ve group had a most powerfull signal. In vivo imaging of annexin V-Cy5.5 signal (f) with removal of head skin or (g) removal of skull bone, and (h) ex vivo imaging of the brain. (i, j) TBRs of annexin V-Cy5.5 showed the Ve group having a most powerfull signal. **p*<0.05 vs SC, #*p*<0.05 vs Ve, scale bar 5 mm.

Figure 3. HE staining of (a) SC group, (b) Ve group, (c) edaravone A and (d) B groups at 48 h after tMCAO. (e) The quantitative analysis of infarct volume, edaravone groups showed a smaller infarct volume in the cerebral cortex and caudate than Ve group. #*p*<0.05 vs Ve, scale bar 3 mm.

Figure 4. Single TUNEL staining of (a) SC group, (b) Ve group, (c) edaravone A (Ed-A) and (d) B (Ed-B) groups at 48 h after tMCAO. (f) Quantitative analysis of TUNEL-positive cells. A strong expression for TUNEL was observed after tMCAO, but edaravone treatments decreased the expression. (e) Western blot analysis of infarct hemisphere for LC3 protein and (g) the quantitative analysis relative to β-tubulin. The amounts of LC3-I and LC3-II (relative to β-tubulin) were significantly higher after

tMCAO, and edaravone treatments significantly decreased those. **p<0.01 vs SC, *p<0.05 vs SC, #p<0.05 vs Ve, scale bar 50 μm.

Figure 5. (a) Antibody for MMP-9 showed an immnohistochemical background as red fluorescent signal in 20 μm sections, which became much stronger in the ischemic hemisphere after tMCAO, and edaravone treatment groups decreased the expression (arrowheads). Double NAGO and MMP-9 of (e) SC group, (f) Ve group, (g) edaravone A (Ed-A) and (h) B (Ed-B). Immunofluorescent study of the ex vivo brain sections (intravenously injected MMPSense 680 or annexin V-Cy5.5) with an antibody for MMP-9 or annexin V showed a (k, n) colocalization of (5j) MMPSense 680 or (m) annexin V-Cy5.5 and exogenous antibody for (i) MMP-9 or (l) annexin V. Western blot analysis of infarct hemisphere for (o) MMP-9, (p) aquaporin 4 and (q, r) the quantitative analysis relative to β-tubulin. The amounts of MMP-9 and aquaporin 4 (relative to β-tubulin) were significantly higher in Ve group, but those in edaravone treated groups significantly decreased than Ve group. **p<0.01 vs SC, *p<0.05 vs SC, #p<0.05 vs Ve, scale bars = 3 mm in a; 50μm in e and k; 5 μm in n.

Figure-1

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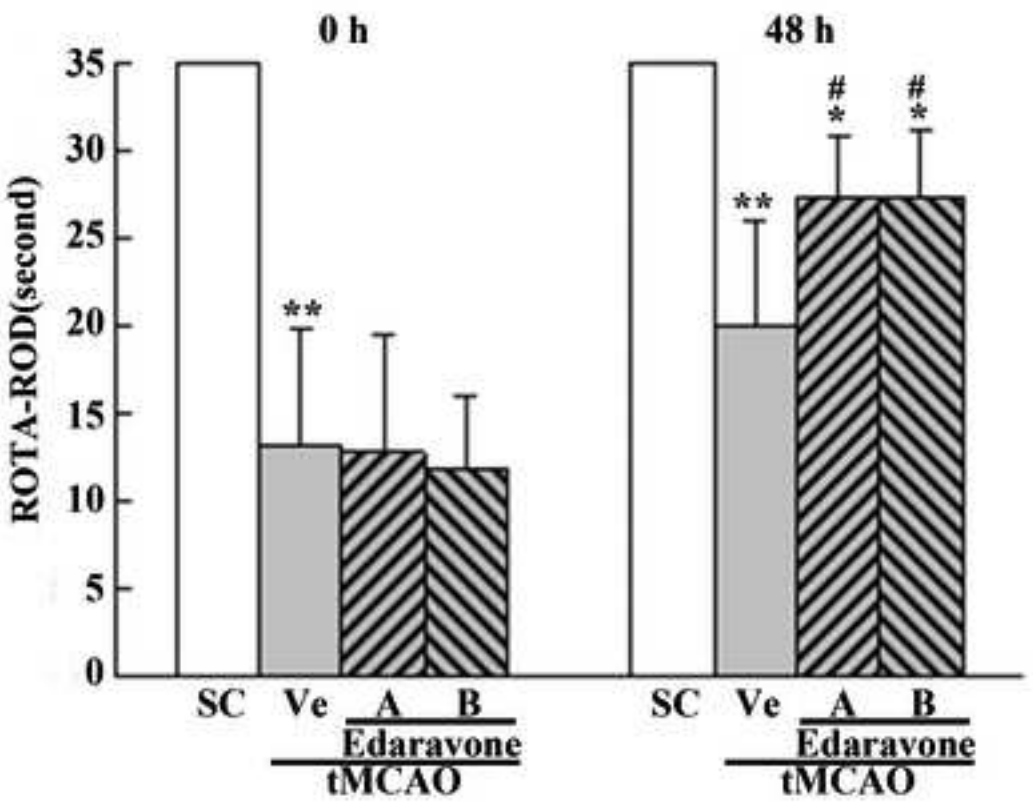

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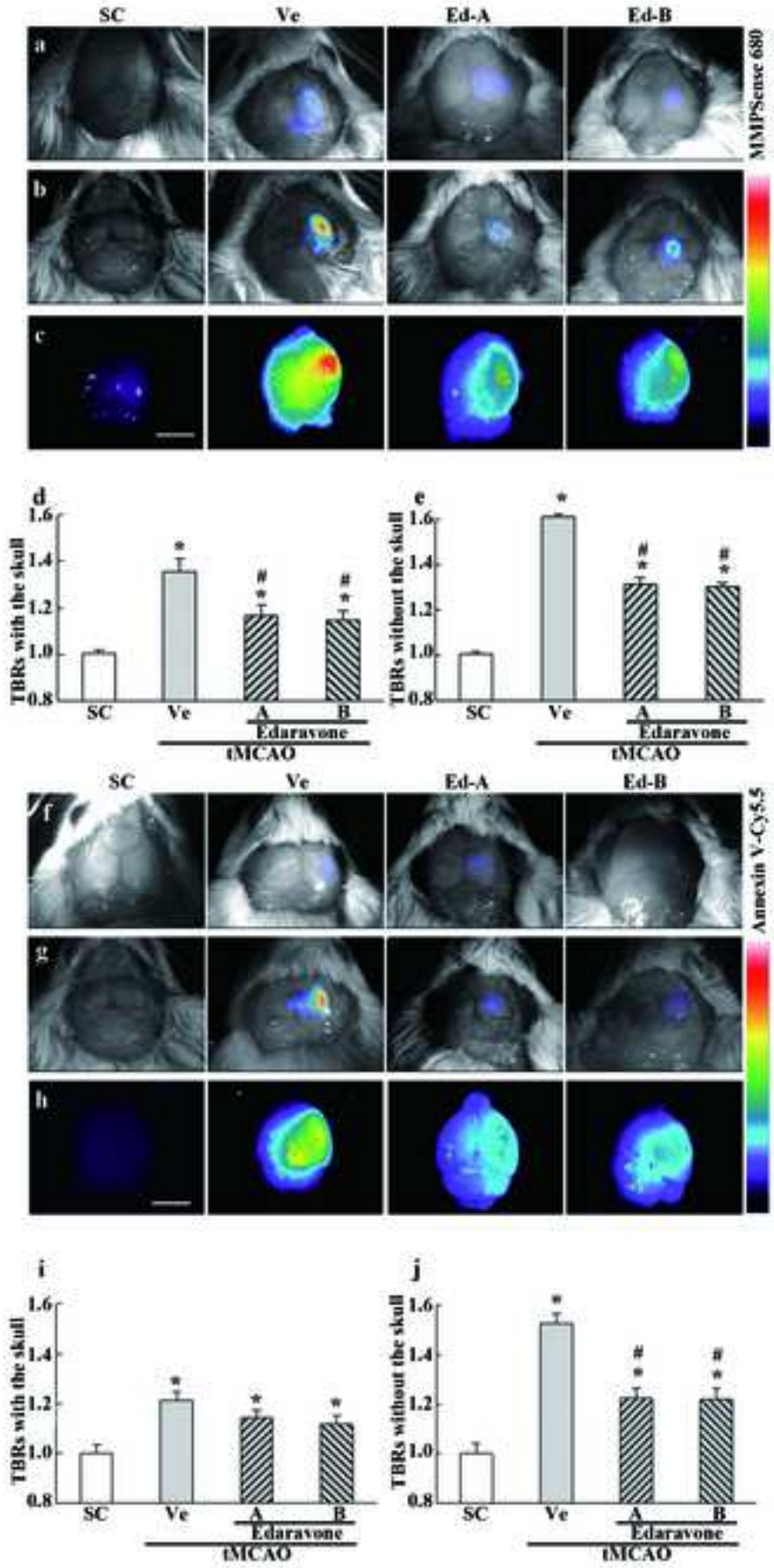

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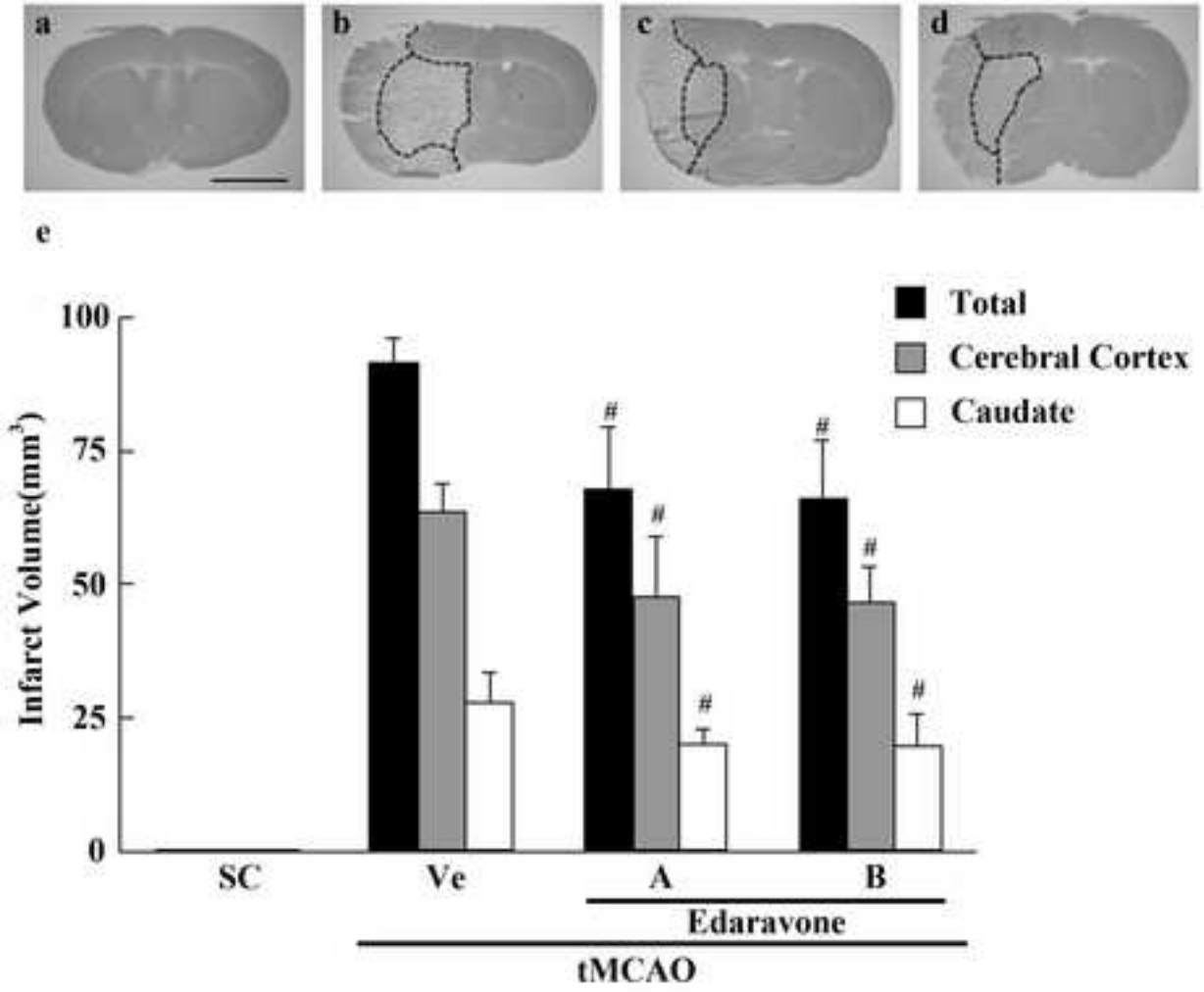

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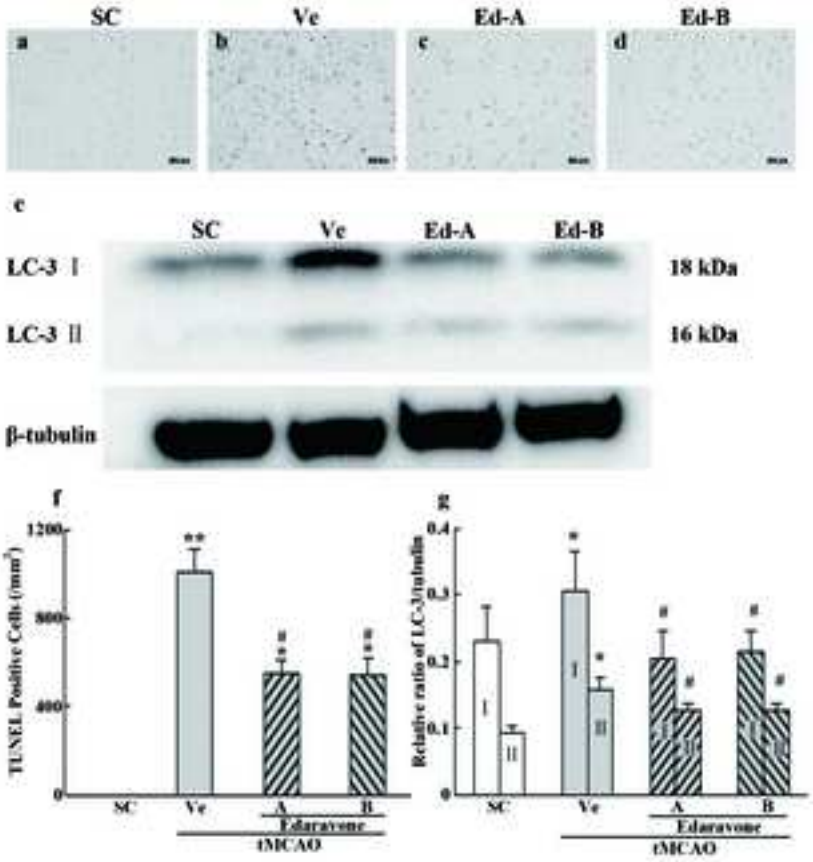

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